Inventory of supplemental information

Four Figures with Figure legends. Figure S1 relates to Figure 1 in the main document; Figure S2-S3 relate to Figure 2 in the main document; Figure 4 relates to Figure 4 in the main document.

The Supplemental experimental procedures go into great details on the physiologic methods, the preparation of the electron microscopy samples and the high-resolution respirometry, as well as the calculations of the quantification of mDNA copy numbers. The antibody table is also included in this section.

SUPPLEMENTAL INFORMATION

Maternal metabolic syndrome programs mitochondrial dysfunction via germline changes across three generations

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (related to Figure 1). Visceral adipose insulin signaling is altered in F1-HF/HS mice. F1 female offspring born to HF/HS-fed (F1-HF/HS) or chow-fed (F1-Con) mothers were weaned onto standard chow diets and assessed at eight weeks of age. Basal insulin signaling was measured in visceral adipose tissue via western blot analysis of tyrosine phosphorylation of IRS1(tyr895) and serine phosphorylation of AKT(ser473) (A). Western blot densitometry for IRS1, P-IRS1 and the ratio of P-IRS1 to IRS1 (B) and P-AKT, AKT, and the ratio of P-AKT to AKT (C) was normalized to the loading control cyclopholin B (cycloB). All data are expressed as mean ± SEM. $*P < 0.05$ by Student's T-test.

Figure S2 (related to Figure 2). Mitochondrial morphology, size and O_2 consumption in F1 skeletal muscle. A. Low magnification TEM images of soleus (Top) and lateral gastrocnemius (Bottom) from F1-Con (left) and F1-HF/HS (right) offspring. Yellow arrowheads denote lipid droplets associated with mitochondria. **B.** Mitochondrial size was determined in soleus (C) and lateral gastrocnemius (D) muscle fibers from F1 mice. **C.** Representative O_2 consumption tracing in permeabilized soleus muscle. **D.** Respiratory control ratio (RCR) in soleus muscle from F1-Con and F1-HF/HS exposed offspring.

Figure S3 (related to Figure 2). MtDNA copy number and mitofusin levels did not differ between F1-Con and F1-HF/HS lateral gastrocnemius muscle Mitochondrial DNA copy number was determined in soleus (**A**) and lateral gastrocnemius (**B**) muscle fibers from F1 mice. **C**. Western blot analysis was used to determine the total protein levels of mitofusins 1 (Mfn1) and 2 (Mfn2) in lateral gastrocnemius muscle from F1 mice. **D.** Densitometry was normalized to the loading control GAPDH. All data are expressed as mean ± SEM.

Figure S4 (related to Figure 4). Mitochondria size is increased in F2 and F3-HF/HS lateral gastrocnemius muscle. Morphological analysis of mitochondria from transmission electron microscopy (TEM) images was used to determine mitochondrial size in F2 (A) and F3 (B) lateral gastrocnemius muscle. All data are expressed as mean ± SEM $*P < 0.05$ by Student's T-test.

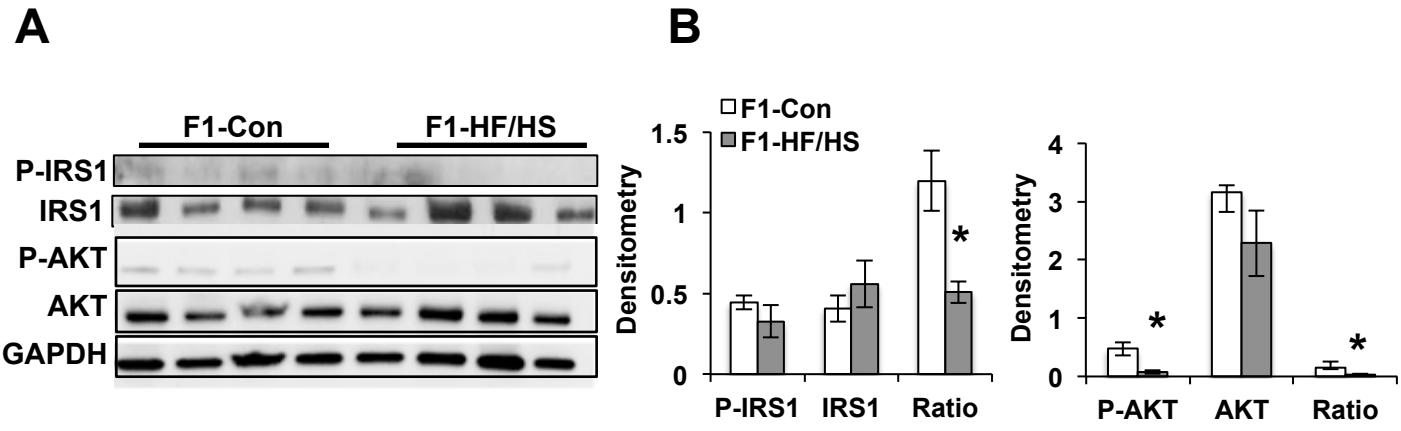
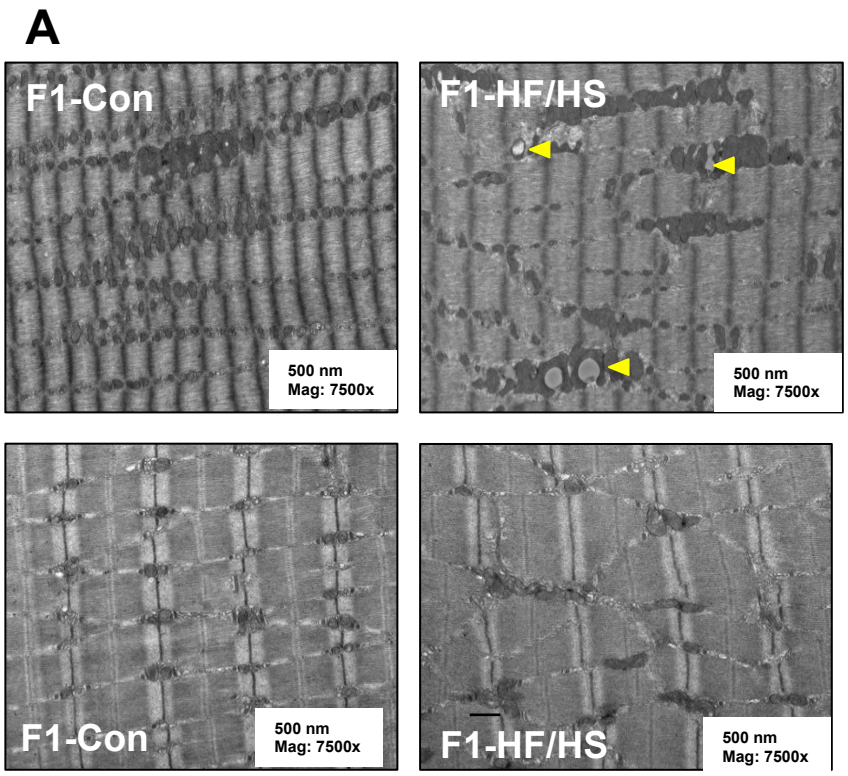
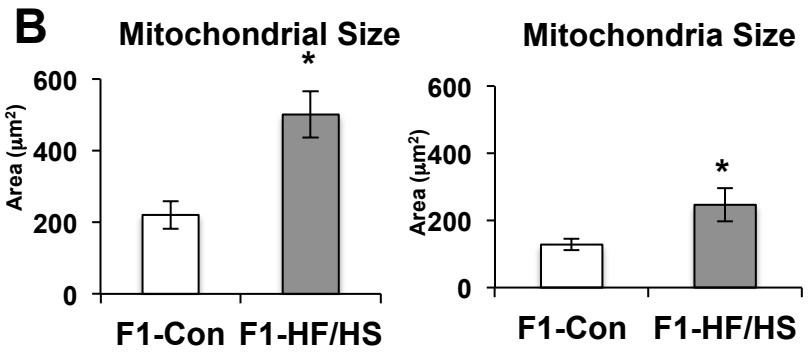
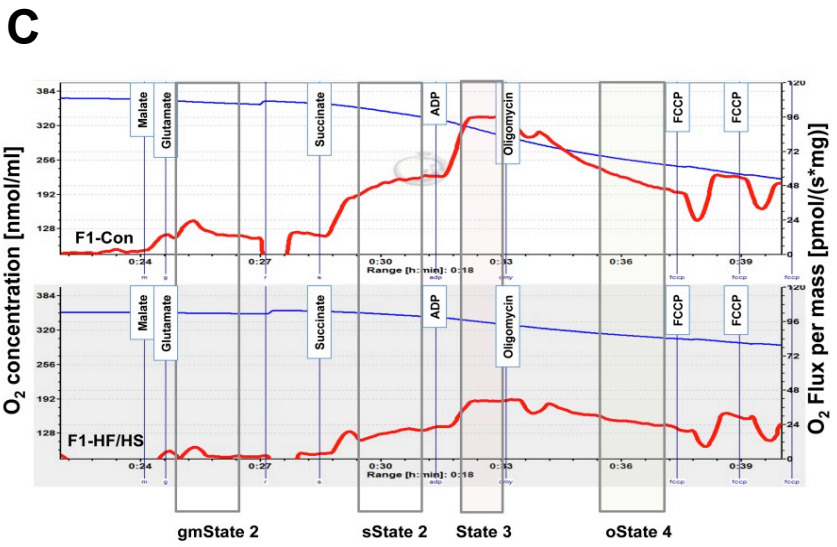


Figure S1 (related to Figure 1)







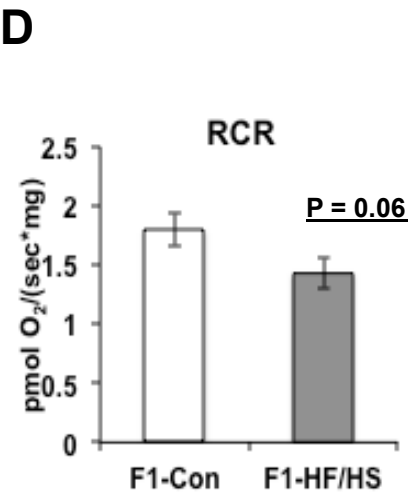


Figure S2 (related to Figure 2)

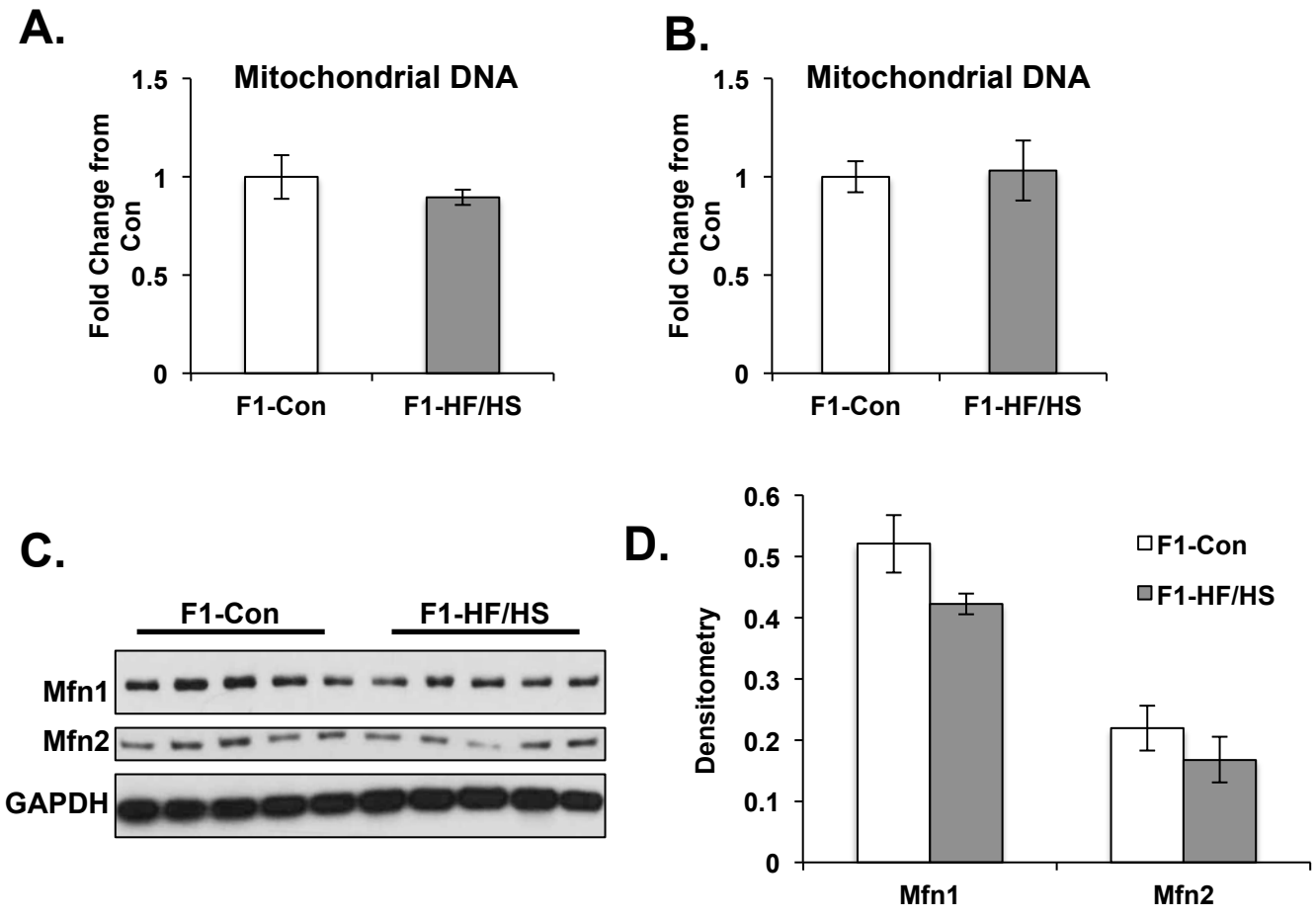


Figure S3 (related to Figure 2)

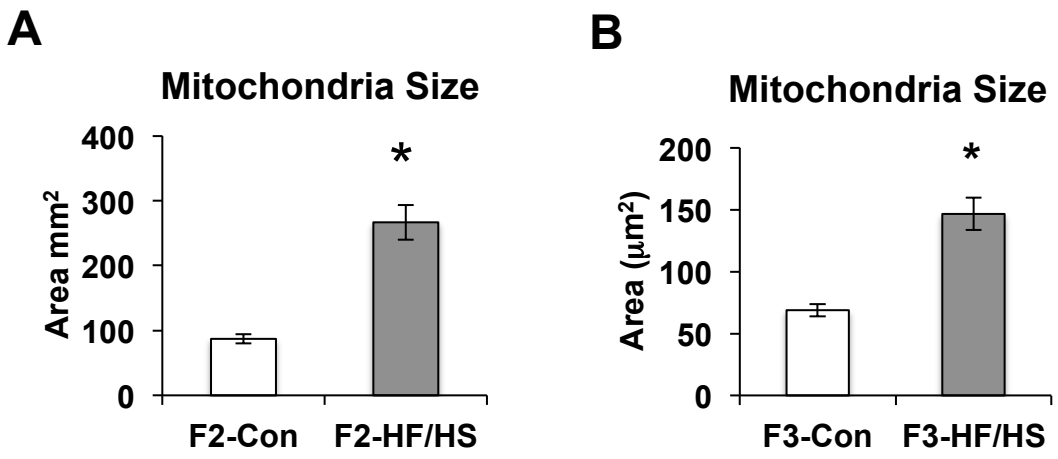


Figure S4 (related to Figure 4)

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Metabolic analysis in F0 mice

After six weeks of high fat-high sugar (HF/HS) (Test Diet 58R3; 59% fat, 26% carbohydrates [17% sucrose] and 15% protein) or standard chow (Con) (PicoLab Rodent diet 20; 13% fat, 62% carbohydrates [3.2% sucrose] and 25% protein) exposure, body weights were measured, body composition was determined by magnetic resonance imaging (EchoMRI 3-in-1, Echo Medical Systems, Houston, TX), and an oral glucose tolerance test was administered following a six-hour fast. Fasting serum was collected and analyzed for circulating triglyceride, cholesterol (Infinity Triglyceride and Cholesterol Reagent kits, Crystal Chem, IL) and insulin (Ultrasensitive Insulin Elisa kit # 90080, Crystal Chem: F0 and F3; Rat/mouse Insulin ELISA kit # EZRMI-13K, EMD Millipore: F1).

Tissue collection

F1, F2, and F3 eight-week-old female mice that were not used for breeding purposes were sacrificed 46-48h after injection of pregnant mares serum (intraperitoneal: 10 IU) and following a 6h fast. At necropsy, serum, visceral adipose (surrounding the gonads), and skeletal muscle (soleus and lateral gastrocnemius) were collected and snap frozen or prepared for transmission electron microscopy as described below. For germinal vesicle (GV)-stage oocyte collection, both ovaries were removed and placed in M2 media + 4 mM hypoxanthine (Sigma-Aldrich, St. Louis, MO). Large antral follicles were punctured with a 29 x ½ gauge needle, and cumulus cells were removed by multiple passes through a glass pipette.

Protein Isolation and Western Blotting

Lateral gastrocnemius muscle was homogenized on ice in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1.0% NP-40, 1.0% deoxycholic acid, 0.1% SDS, and 2 mM EDTA) containing 1 mM PMSF and protease inhibitor cocktail (Aprotinin, Leupeptin, Peptstatin, AEBSF, PIC1, PIC2). Protein concentration was measured with the Pierce BCA protein assay (Fisher #PI-23227), and samples containing 25 ng of total protein were separated on 4-20% SDS-polyacrylamide gels (Bio Rad #4561096), transferred to a 0.2 mm nitrocellulose membrane (Bio-Rad #162-0112), and probed with primary antibodies (see antibody table below) for 16 h at 4 °C. Blots were washed and then probed with HRP-conjugated anti-rabbit and anti-mouse IgG (Santa Cruz Biotechnology). The SuperSignal West Pico or West Femto kit was used to detect signal, and ImageJ-Fiji software was used to quantitate band intensities on film.

Antibody Table

Peptide/protein target	Name of Antibody	Manufacturer, catalog #	Species raised in; monoclonal or polyclonal	Dilution
Phospho-AKT(Ser473)	P-AKT	Cell Signaling, #4060	Rabbit, monoclonal	1:1000
AKT	AKT	Cell Signaling, #4691	Rabbit, monoclonal	1:1000
Cyclophillin B	CycloB	Abcam, #ab16045	Rabbit, polyclonal	1:5000
Phospho-DRP1(Ser616)	P-Drp1	Cell Signaling, #355S	Rabbit, polyclonal	1:1000
DRP1	Drp1	Cell Signaling, #8570S	Rabbit, monoclonal	1:1000
GAPDH	GAPDH	Cell Signaling, #2118L	Rabbit, monoclonal	1:5000
Phospho-IRS-1(Tyr895)	P-IRS1	Cell Signaling, #3070S	Rabbit, polyclonal	1:1000
IRS1	IRS1	Cell Signaling, #2382S	Rabbit, polyclonal	1:1000
Mitofusin 1	Mfn1	Abcam, #ab57602	Rabbit monoclonal	1:1000
Mitofusin 2	Mfn2	Cell Signaling, #9482S	Rabbit monoclonal	1:1000
OPA1	OPA1	BD Bioscience, #612606	Mouse, monoclonal	1:1000

Transmission Electron Microscopy

Soleus, lateral gastrocnemius, and GV-stage oocytes were fixed (2% paraformaldehyde + 2.5% glutaraldehyde in cacodylate buffer) and embedded in 2% agarose. Agarose blocks were fixed in 1% OsO4 (Electron Microscopy Sciences, Hartfield, PA), stained in 1% uranyl acetate, and dehydrated in a series of ethanol washes. Next, agarose-embedded tissues were washed twice in propylene oxide (Electron Microscopy Science) and then infiltrated with resin by using the Eponate 12 Kit (Ted Pella, Redding, CA). After infiltration, blocks were embedded, cured, and then sectioned with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL). Sections (90 nm) were stained with uranyl acetate and Reynolds' lead citrate before viewing at 3,000x - 20,000x magnification with a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA) equipped with an AMT 8-megapixel digital camera (Advanced Microscopy Techniques, Woburn, MA). Images were analyzed by three independent, blinded observers for mitochondrial size, mitochondrial shape, and the number of lipid droplets in each image.

Preparation of permeabilized muscle fibers and high-resolution respirometry

After excision, soleus muscles were immersed in cold BIOPS (10 mM EGTA, 50 mM MES, 0.5 mM DTT, 6.56 mM $MgCL_2$, 5.77 mM ATP, 20 mM Imidazole, 15 mM phosphocreatine, pH 7.1) until fiber preparation. After all samples were collected, fibers were separated on ice by using two sharp-tipped forceps under a dissection microscope. Separated fibers were permeabilized with BIOPS solution containing 50 μg/mL saponin for 30 minutes at 4 °C. Next, fibers were washed for 10 minutes in ice-cold mitochondrial respiration solution (MIR05, 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurin, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA, pH 7.1). Fibers were weighed, blotted dry (3-5 mg total tissue weight) and placed in an Oxygraph 2K (OROBOROS Instruments, Innsbruck, Austria) chamber containing 2 mL of 37 °C MirO5. To measure O_2 flux, the following substrates were added sequentially: 5 mM malate, 10 mM glutamate, 10 mM succinate, 2.5 mM ADP, 2 μg/mL Oligomycin, followed by 3 pulses of 0.5 μM FCCP. A period of stabilization followed the addition of each substrate, and DatLab Software (OROBOROS Instruments, Innsbruck, Austria) was used to record oxygen flux per mass.

Mitochondrial DNA abundance

To extract genomic DNA, soleus and lateral gastrocnemius muscle were homogenized in DNA extraction buffer (50 mM Tris, 1mM EDTA, 0.5% SDS, 1 mg/ml Proteinase K, pH 8.0) and incubated 12-16h at 55 °C. Genomic DNA was extracted with phenol/chloroform in 5 Prime Phase Lock Gel tubes (Fisher Scientific #FP2302820), and RNA was degraded by incubating for 1h in 10 mg/ml RNaseA at 37 °C. An additional phenol/chloroform extraction was performed, and genomic DNA was precipitated by adding 1/10 of the sample volume of 3M NaAcetate (pH5.2) and 2.5 volumes 100% EtOH. DNA was washed in 70% ETOH and resuspended in 50mμl of TA (10mM Tris, 0.5mM EDTA, pH 8.0). Genomic DNA was quantified by using Qubit Fluorometric Quantitation (Thermo Fisher Scientific). One microgram of DNA was used for quantitative real-time PCR using the ABI system and mouse nuclear (H19: forward-GTACCCACCTGTCGTCC, reverse- GTCCACGAGACCAATGACTG) and mtDNA (ND1: forward-CTCTTATCCACGCTTCCGTTACG, reverse-GATGCTGGTACTCCCGCTGTA) primers. A version of delta CT was used to quantify mitochondrial abundance:

$$2^{([Ct(A)*E(A)]-[Ct(B)*E(B)])}$$

where Ct= cycle threshold, E= efficiency, A = ND1 and B = H19

A standard curve was generated for both genes by analyzing a 10-fold dilution series to determine the efficiency of both reactions (value out of 100%). Values were then expressed as fold change from the Con values.

mtDNA copy number in GV oocytes

Single oocyte was loaded in a PCR tube with 10 µl lysis buffer (50 mM Tris-HCL, 0.1mM EDTA, 0.5% Tween-20, 200 mg/ml Proteinase K, pH 8.5) and incubated at 55 °C for 2 h. Proteinase K was heat inactivated at 95 °C for 10 min, and then the samples were used directly for PCR analysis. Quantitative real-time PCR was performed by using the ABI system and mouse mtDNA-specific primers: B6 forward, AACCTGGCACTGAGTCACCA, and B6 reverse, GGGTCTGAGTGTATATATCATGAAGAGAAT. To obtain standard curves, standard DNA was prepared by ligating amplified PCR products (B6-forward and B6-reverse) into the TOPO vector by T/A cloning. Plasmid concentrations were measured by using Nanodrop, and DNA copy number was calculated at http://www.uri.edu/research/gsc/resources/cndna.html. Extracted plasmid was diluted to 10^8 copies per microliter and then serial diluted (from 10^6 to 10^2 copies per microliter).